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SUFFIELD MEMORANDUM

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RAPID IMMUNOFILTRATION ASSAY OF FRANCISELLA TULARENSIS

by

H. Gail Thompson and William E. Lee

PCN 351SQ

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ACKNOWLEDGEMENTS

We would like to thank John Cherwonogrodzky for his helpful discussions during the course of this work and critical review of the manuscript.

ABSTRACT

A rapid immunofiltration assay for a killed bacterial preparation of *Francisella tularensis* was developed. Formalin treated cells of *Francisella tularensis* were incubated with polyclonal antibody to *Francisella tularensis* and an anti-species antibody conjugated to urease. The reaction mixture was filtered through albumin coated 0.45 μm nitrocellulose membranes following incubation periods which varied from 1 to 60 min. The pH sensing capability of the detector, a light-addressable potentiometric (LAP) sensor, was employed to detect the presence of immobilized urease-conjugated antibodies. The time required for the assay was about 5 min exclusive of the incubation period. Limits of detection (LOD) of the assay, determined as a function of the incubation time were 1.2×10^5, 5×10^4 and 3.4×10^3 cells for incubations of 1,5 and 60 min. The speed and ease of the assays suggest that the LAP sensor technology is suitable as a detection system for a mobile field laboratory.

RÉSUMÉ

On a mis au point une méthode de dosage par immunofiltration rapide qui s'applique à des préparations bactériennes de *Francisella tularensis* tuées. Des cellules de *Francisella tularensis* traitées à la formaline ont été incubées avec un anticorps polyclonal dirigé contre *Francisella tularensis* et un anticorps anti-spécifique conjugué à l'uréase. Àprès une incubation variant de 1 à 60 minutes, le mélange réactif a été filtré sur des membranes de nitrocellulose (0,45 μm) enduites d'albumine. La capacité de détection du pH propre au détecteur, un capteur potentiométrique adressable par la lumière (CPAL), a été mise à profit pour déceler la présence d'anticorps immobilisés conjugués à l'uréase. Le dosage peut être réalisé en 5 minutes environ, sans compter la période d'incubation. Les limites de détection déterminées en fonction de la durée de l'incubation, étaient de 1, 2 x 10^5, 5 x 10^4 et 3, 4 x 10^3 cellules pour des périodes d'incubation de 1,5 et de 60 minutes. La rapidité et la facilité de réalisation de la méthode laissent croire que le capteur potentiométrique adressable par la lumière constitue un système approprié de détection pour le laboratoire mobile de campagne.

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INTRODUCTION

Advances in molecular biology have augmented the potential of pathogens to be deployed as BW agents. The detection of pathogens presents a major challenge to the bioanalytical capabilities of defence research. Effective protection and therapy against BW agents rest on the ability to detect and identify these threats.

Francisella tularensis, a gram-negative bacterium responsible for tularemia, has been reported in North America, Europe, Japan and Russia (1-4). Although it is largely a disease of wildlife, especially rabbits, the zoonotic nature of the bacterium make it a threat to human health (5). Clinical manifestations have been classified based on portal of entry: ulceroglandular, glandular, oculoglandular, oroglandular, oropharryngeal, typhoidal and pneumonic (6). The treatment of choice for tularemia is streptomycin sulfate (6); kanamycin sulfate, gentamicin, tetracycline hydrochloride and choramphemicol have been shown effective (7,8).

Owing to the BW threat posed by bacterial pathogens, the Canadian Forces have formulated a need for a mobile field laboratory for the identification and quantitation of pathogens and threat agents. The ideal assay system for a mobile field laboratory would be sensitive, rapid, and adaptable to a wide range of threat agents. In addition, other desirable features would include compact physical size, ruggedness (deriving from a limited number of mechanical parts) and an ease of operation with regard to the amount of time and skill required by the operator to conduct assays. A technology having strong potential for defence related bioanalysis is the light-addressable potentiometric (LAP) sensor (Figure 1) (9). The sensing component of the device is a silicon semiconductor chip which is able to monitor changes in pH caused by enzymes such as urease and penicillinase. Coupled with rapid filtration capture, and used in conjunction with enzyme immunoassay technology, the LAP sensor is a useful detection system and is able to meet

the above criteria. The LAP sensor is employed in the biosensor module of the BioChemical Detector (BCD) and has proven successful in the quantitative analysis of simulants for virus, toxin and bacteria threat agents (10-12).

This work presents an example of an assay on a LAP sensor for a killed-cell bacterial preparation, *Francisella tularensis.* The rapid quantitative immunofiltration assay described in this report was undertaken to assess the feasibility of employing the LAP sensor-based instrument for a mobile field laboratory. Formalin treated cells of *Francisella tularensis* were incubated with polyclonal antibody to *Francisella tularensis* and an anti-species antibody conjugated to urease. The cells were captured by filtering reaction mixture through albumin coated 0.45 μm nitrocellulose membranes. The time required for processing a sample in the assay system was about five minutes, exclusive of the incubation period. Limits of detection (LOD) of the assay, determined as a function of the incubation time were 1.2×10^5, 5×10^4 and 3.4×10^3 cells for incubations of 1, 5 and 60 min.

MATERIALS AND METHODS

Bovine serum albumin (BSA), sodium chloride, sodium dihydrogen phosphate, Tween 20, Triton X-100, phosphate buffered saline (PBS) anti-rabbit IgG (goat) urease conjugate, and urea were purchased from Sigma Chemical Co. (St Louis, MO) and used without further purification. Streptavidin was obtained from Scripps Laboratories (San Diego, CA). It was reconstituted in distilled water to yield a stock concentration of 10 mg/mL. Polyclonal antibodies to *Francisella tularensis* from rabbit (purified by caprylic acid and ammonium sulfate precipitations) were a generous gift of the Molecular Biology Group, DRES. Immunoelectrophoresis performed by the Molecular Biology Group indicated a high IgG content. Protein concentration of the antibody preparation was determined spectrophotometrically to be 3.1 mg/mL by means of a BCA protein assay kit (Pierce Chemical Co., Rockford, IL) and bovine plasma IgG standards (BioRad, Mississauga, ON). The antigen, *Francisella tularensis*, LVS strain, was acquired from U. S. Army Medical Research Institute for Infectious Diseases (Frederick, MD), deactivated with 0.5% formaldehyde and stored as a lyophylized powder. It was reconstituted in PBS and sonicated (Soniprep sonicator model 150, power setting 4) for four cycles of 15 sec bursts followed by 45 sec cooling on ice.

For the immunofiltration assays, wash solution consisted of 150 mM NaCl, 10 mM phosphate buffer, pH 6.5, plus 0.2% Tween 20 detergent. The dilution buffer was the wash solution titrated to pH 7.0, containing 1% BSA and 0.25% Triton X-100. The substrate solution for the enzyme assays was wash solution containing 100 mM urea.

Apparatus

The apparatus for these assays was a commercially available LAP sensor, marketed under the name Threshold Unit™. It was purchased from the manufacturer, Molecular Devices Corp. (Menlo Park, CA). The instrument was controlled by an IBM PS/2 model 30 microcomputer and custom designed software supplied by Molecular Devices Corp. The design of the Threshold Unit allowed eight samples to be tested simultaneously. Nitrocellulose membrane filters (0.44 μm pore size) coated with BSA were purchased from Molecular Devices Corp.

Immunofiltration Assay

Figure 2 depicts the scheme of an immunofiltration assay for *Francisella tularensis*. Reagent solutions consisted of 3 μL anti-*Francisella tularensis* and 10 μL anti-rabbit IgG in 10 mL diluent solution. An aliquot (150 μL) of reagent solution was added to each sample (100 μL) of killed suspended cells, mixed thoroughly and allowed to incubate at room temperature. After the required incubation time had elapsed, a portion (150 μL) of the reaction mixture was delivered to a well of the filtration assembly and filtered through the membrane at 250 μL min^{-1}. After the filtration was completed, 500 μL of wash buffer was added to the well and the rate of filtration was increased to 750 μL min^{-1}.

The membrane stick containing the immobilized antigen-antibody complexes was removed from the filter assembly and inserted into reader compartment of the Threshold Unit which contained the LAP sensor and the substrate solution. The membrane was pressed against the silicon surface of the sensor by a mechanical plunger. The apparatus was constructed so that the spots (3 mm dia.) on the membrane which contained the immobilized antigen/antibody complexes aligned with the sensing areas on the silicon chip. Hydrolysis of urea to carbon

dioxide and ammonia at the membrane-sensor surface interface resulted in an increase of pH which was detected as a change in surface electropotential. The membrane was monitored for enzyme induced pH change for one minute. The data was collected and stored on the microcomputer using software designed by Molecular Devices Corp. The rate of change of pH at the surface of the silicon sensor was monitored by the rate of change with respect to time of the surface potential as μv/sec.

RESULTS

Assays of *Francisella tularensis* were carried out with incubation times of 1, 5 and 60 min. The results of a 1 min incubation assay of *Francisella tularensis* are presented in Figure 3. The standards ranged from 100 to 1500 ng per well and were run simultaneously on a single membrane stick. Each data point represents the mean of three consecutive assays performed with the same reagents. The data, μv/sec versus ng of antigen was well represented by a linear plot ($r^2 = 0.998$). The errors associated with the individual points on the calibration curve were about 10%. The LOD taken to be the intersection of the calibration curve with the mean background (output of the LAP sensor for reagents only, zero *Francisella tularensis* antigen) plus two standard deviations (SD) of the mean background was about 130 ng per well or 1.7×10^5 cells per well (based on a weight of 8×10^{-13} g per cell (20)).

A 5 min incubation assay of *Francisella tularensis* was carried out in a similar manner with standards ranging from 75 to 1200 ng per well (Figure 4). Employing the criterion as above, the LOD was determined to be about 56 ng (7.5×10^4 cell) per well. For a sixty minute assay (Figure 5), a LOD of 3.8 ng (5.1×10^3 cells) was obtained. In terms of relative detectablity, the assays of 5 and 60 min displayed an increase of 2.3- and 34-fold, respectively. The results are summarized in Table I.

Quantitations of *Francisella tularensis* were carried out by presenting samples to the LAP sensor. The recovered values of *Francisella tularensis* were on average within 4% of the spiked amounts. A linear regression (Figure 6) yielded a high correlation ($r^2 = 0.9996$) between spiked and recovered quantities.

DISCUSSION

In the assay for *Francisella tularensis*, bacterial cells were captured onto solid phase nitrocellulose membrane by filtration. The time required for immobilization of bacterial cells via filtration was approximately 1-2 min, a significant reduction over that of microtiter plates (typically 1 h) wherein the rate of immobilization is governed by diffusion or gravity. The immunocomplexes of antibody reagents and antigen were formed in a one-step homogeneous incubation further reducing the number of steps and the total time required per assay. In practice the time per assay was about 5 min plus the time allotted for incubation. In addition, the solid support nitrocellulose membranes required none of the pre-assay preparation required for microtiter plates, namely capture antibody coating and blocking. The LOD of a 5 min incubation immunofiltration assay for *Francisella tularensis* proved to be as low as that for a 4 h colorimetric microtiter plate assay (13).

The immunoreagents employed for the assay were an anti-*Francisella tularensis* polyclonal IgG (rabbit) and a commercially available anti-rabbit IgG covalently linked to urease. No chemical modifications, conjugations or purifications of antibodies were required on the part of the user. The LAP sensor is adaptable to other antigens including viruses and proteins. For noncellular antigens, immobilization onto nitrocellulose is accomplished through biotin-streptavidin interactions. Assays of Newcastle Disease Virus and mouse immunoglobulin G yielded LODs of about 3 ng and 10 pg, respectively, for incubations of 1 h (10,11).

The detection system described in this work is fully capable of providing rapid clinical diagnosis of bacterial diseases, such as tularemia in the field. Enzyme-linked immunosorbent assays (ELISAs) for serodiagnosis of tularemia have been developed for anti-*Francisella tularensis* antibodies (14), whole cells (15,16), bacterial antigens (14,17), lipopolysaccharides (3,14) and outer membrane proteins (16,18,19). On the LAP sensor, assays of these clinical indicators can be completed within a period of about thirty minutes.

The physical dimensions of the LAP sensor system and the associated apparatus are about 0.05 m^3 (2 cu. ft) and 7 kg (15 lb). It is a commercially available unit with relatively few moving parts and as a consequence is rugged and durable. The principal mechanical features are a vacuum pump to facilitate filtration and a plunger to press the membrane against the surface of the silicon chip. The sensor chip, itself, is contained in a plastic compartment (10 x 10 x 3 cm) and can be considered to be a consumable item, replaced at regular intervals (2 to 8 weeks).

The LAP sensor is the detection system of the BioChemical Detector (BCD). Since the BCD is a self-contained automated detector, the physical shape and size are different than the apparatus employed here and the final BCD model would most likely not be suitable as a laboratory device. Nevertheless, the homology of these systems is great, especially for the immunoreagents. Advances made in procuring and screening of antibodies for the BCD project are available *gratis* to a field detector. Furthermore, if the BCD is to function as a remote alarm system, a complementary identification and quantitation would be required in a field laboratory and there are definite advantages for employing the same immunoassay format at the remote and mobile sites. The assays described in this report were performed in a laboratory environment in the absence of contaminants or interfering agents. The object of this report was to demonstrate that the LAP sensor detection system, combined with enzyme immunoassay technology has potential for mobile field laboratory applications.

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Table I. Summary of Results of Immunofiltration Assay

incubation time	LOD		Relative Detectability
min	ng	cells[1]	
1	130	1.5×10^5	1.0
5	56	7.5×10^4	2.3
60	3.8	5.1×10^3	34

[1]based on a weight of 8×10^{-13} g per cell (20).

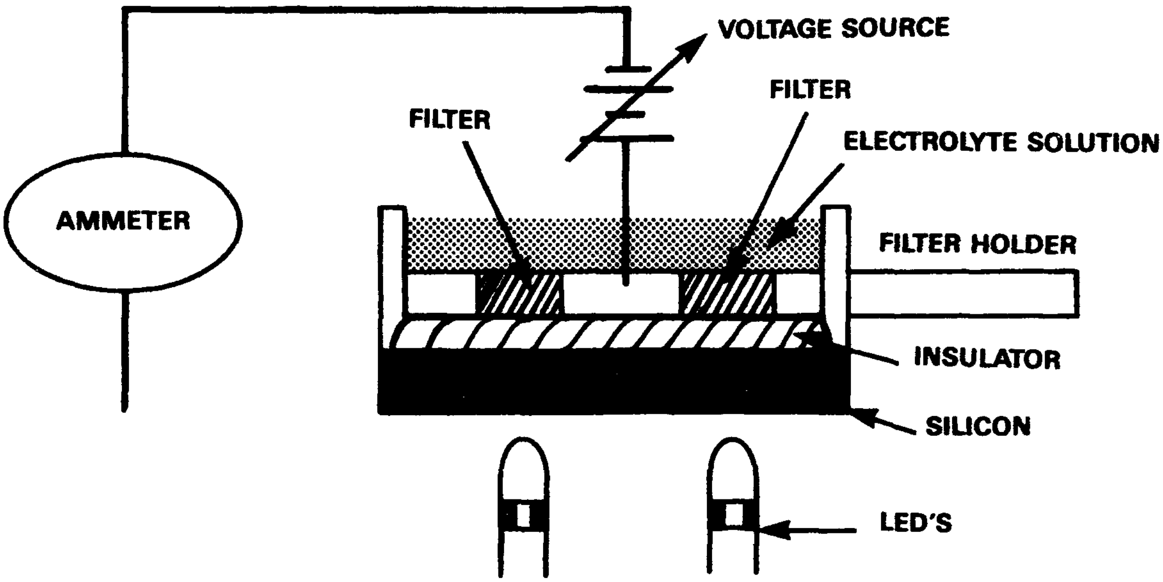

Figure 1

SCHEMATIC DIAGRAM OF SILICON SENSOR DESCRIBED BY HAFEMAN ET AL. (9). THE SILICON IS COVERED WITH AN INSULATOR WHICH SEPARATES IT FROM THE ELECTROLYTE SOLUTION CONTAINING ENZYME SUBTRATE. THE ELECTRICAL POTENTIAL ON THE SURFACE OF THE INSULATOR (LOCATED UNDER THE FILTERS) IS MEASURED VIA A LIGHT-GENERATED ALTERNATING CURRENT. BY ELIMINATING THE LIQUID ABOVE THE MEMBRANE WITH A PLUNGER (NOT SHOWN), REDUCED VOLUME AND THUS GREATER SENSITIVITY ARE ACHIEVED. LED, LIGHT-EMITTING DIODE. AFTER LIBBY AND WADA, J. CLIN. MICROBIOL., 27, 1456-1459 (1989).

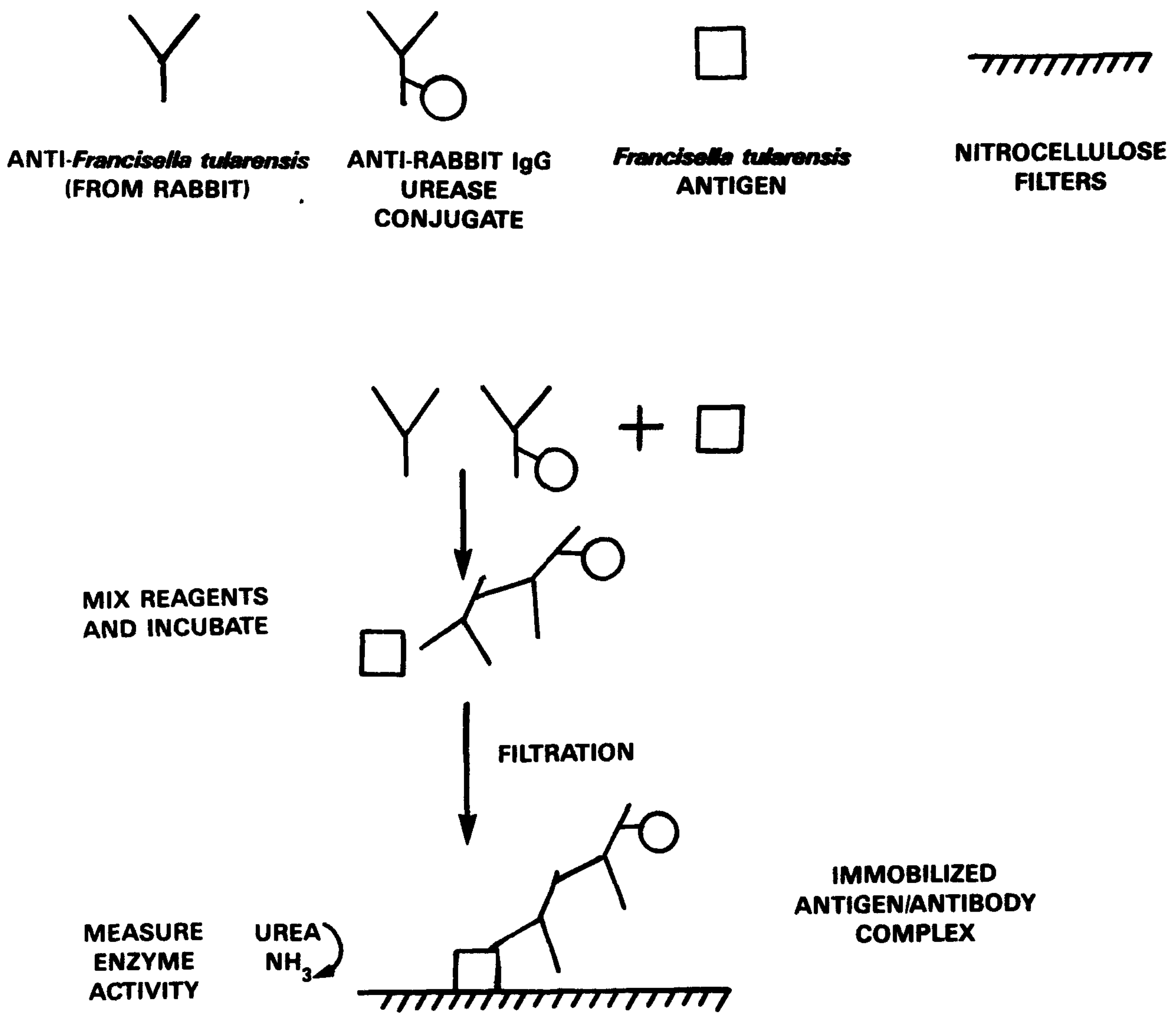

Figure 2

REACTION SCHEME OF THE LAP SENSOR IMMUNOFILTRATION ASSAY.

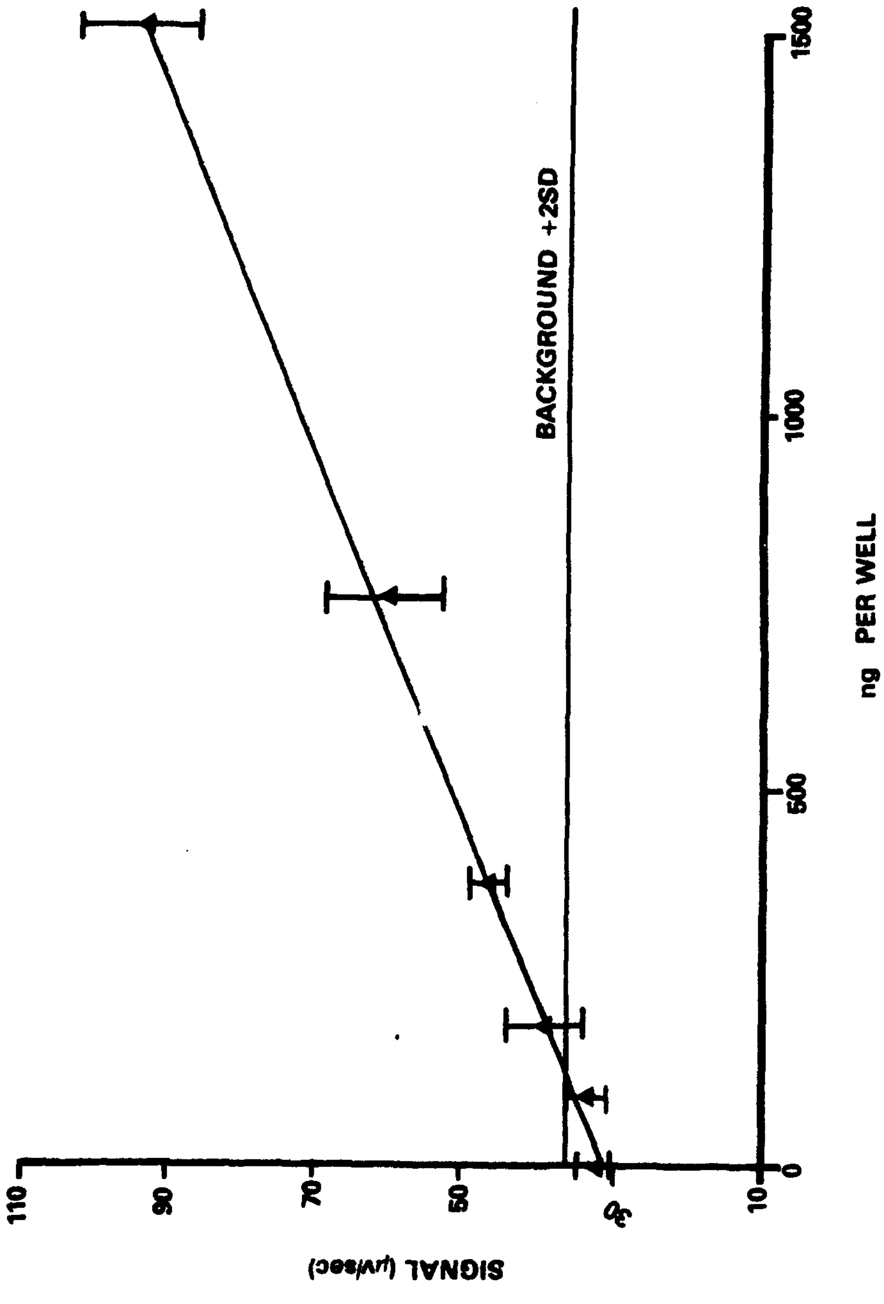

Figure 3

1 min ASSAY OF *Francisella tularensis*. THE ERROR BARS REPRESENT ±S.D. (n = 4, r^2 = 0.998)

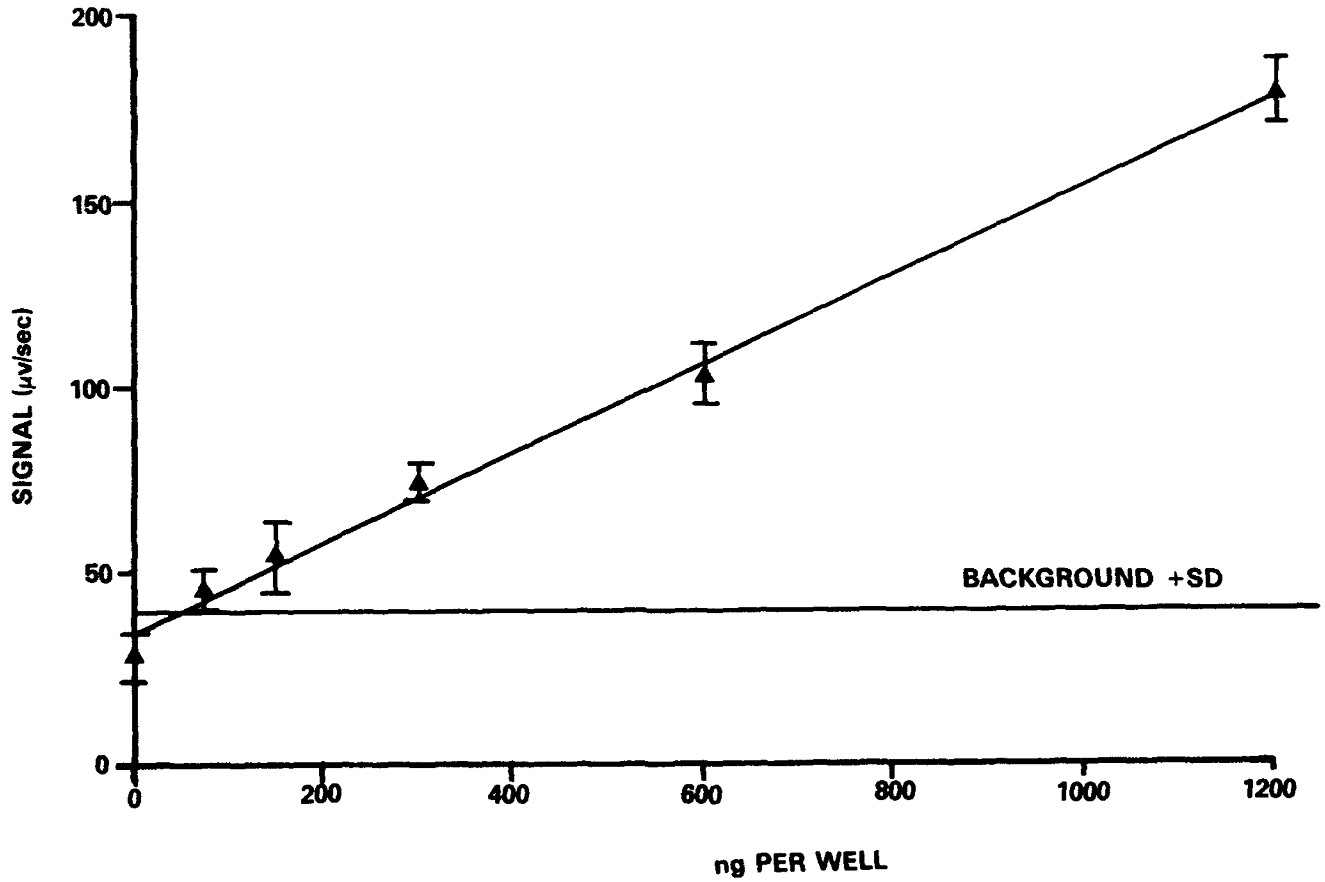

Figure 4

5 min ASSAY OF *Francisella tularensis*. THE ERROR BARS REPRESENT ±S.D. (n = 4, r^2 = 0.998).

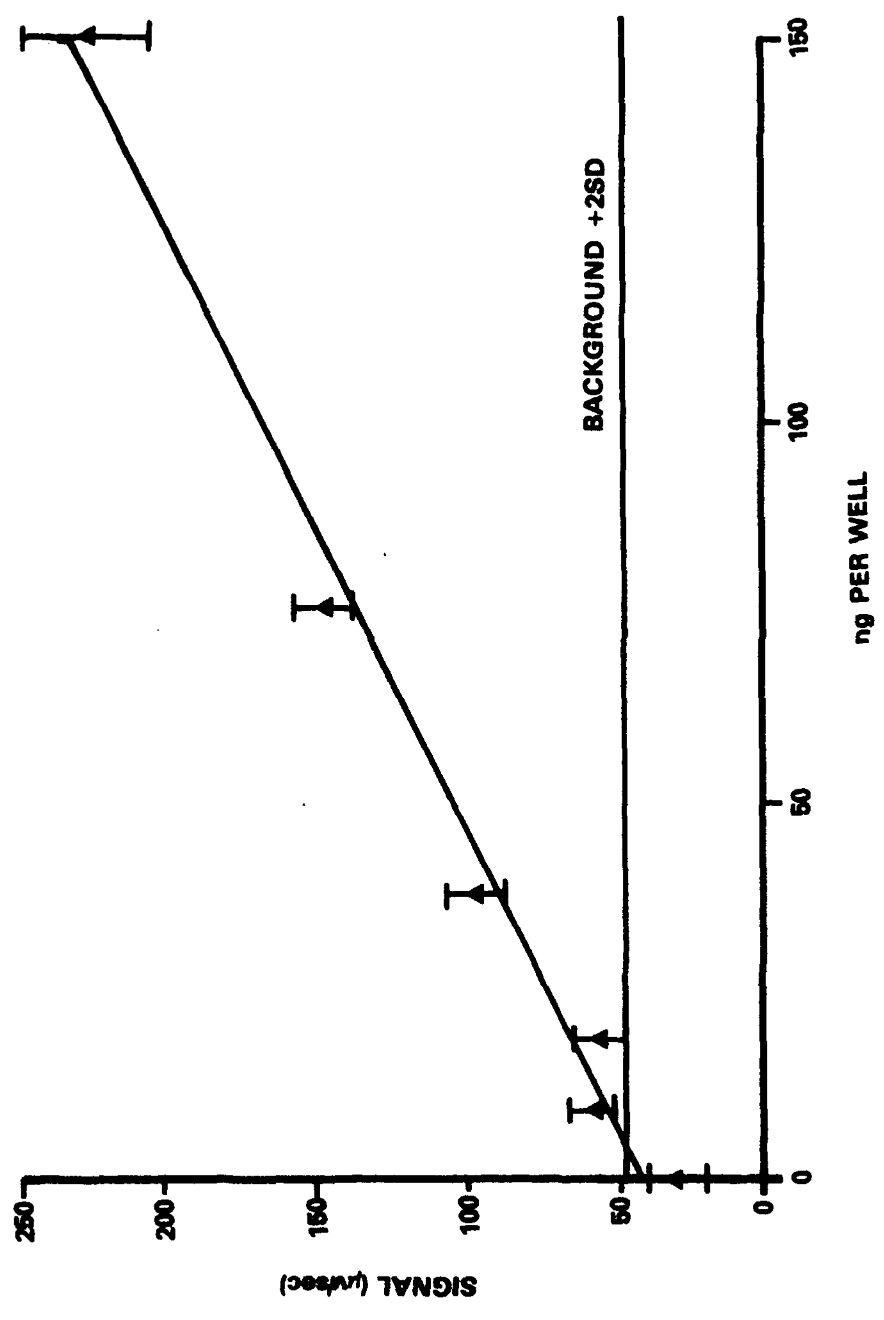

Figure 5

60 min ASSAY OF *Francisella tularensis.* THE ERROR BARS REPRESENT ±S.D. (n = 4, $r^2 = 0.991$)

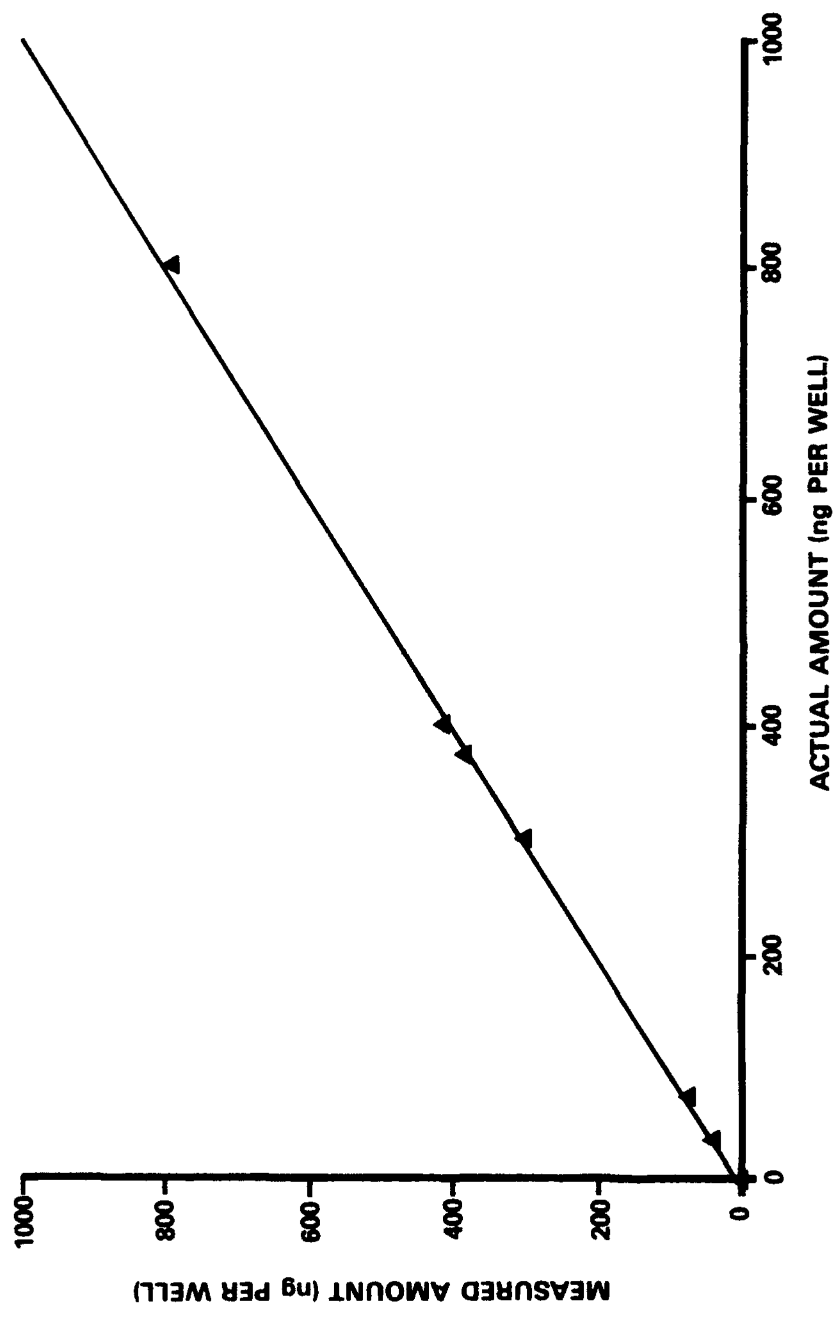

Figure 6

QUANTITATION OF UNKNOWN SAMPLES OF ***Francisella tularensis*** (r^2=0.999).

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A rapid immunofiltration assay for a killed bacterial preparation of Francisella tularensis was developed. Formalin treated cells of Francisella tularensis were incubated with polyclonal antibody to Francisella tularensis and an anti-species antibody conjugated to urease. The reaction mixture was filtered through albumin coated 0.45 µm nitrocellulose membranes following incubation periods which varied from 1 to 60 min. The pH sensing capability of the detector, a light-addressable potentiometric (LAP) sensor, was employed to detect the presence of immobilized urease-conjugated antibodies. The time required for the assay was about 5 minutes exclusive of the incubation period. Limits of detection (LOD) of the assay, determined as a function of the incubation time were 1.2×10^5, 5×10^4, and 3.4×10^3 cells for incubations of 1, 5, and 60 minutes. The speed and ease of the assays suggest that the LAP sensor technology is suitable as a detection system for a mobile field laboratory.

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